SOME EFFECTS OF AMMONIA ON PLANT METABOLISM AND A POSSIBLE MECHANISM FOR AMMONIA TOXICITY[1, 2, 3, 4]

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Plant injury has been traced to ammonia derived from organic sources such as urine (9), chicken manure (8), cottonseed meal (12) and animal manure (3). In addition, inorganic sources such as aqua ammonia, anhydrous ammonia, and ammonium salts have been reported injurious to plants when placed too close to the root zone (3, 7). The toxicity of ammonia to animals has been demonstrated by Sumner (10). The injection of urease into rabbits resulted in death of the animals when NH_3 in the blood rose to 5 mg per 100 ml and before any change in blood pH could be detected. Ammonia and ammonium compounds are being used as fertilizers in increasing amounts. Therefore the question of how ammonia produces the toxic symptoms which frequently result from its use on plants becomes a matter of practical as well as theoretical interest.

The object of this work was to study the effect of ammonia on plant metabolism with the view of locating one or more sites at which ammonia could be shown to have a deleterious effect on the normal metabolic processes of plants.

MATERIALS AND METHODS

Respiratory rates of intact plant tissue and of mitochondria, in the presence and absence of ammonia and ammonium salt, were determined in a Warburg respirometer at 25° C.

Plant materials used included roots of barley, *Hordeum sativum* Pers., leaf and root disks of sugar beet, *Beta vulgaris* L. var. and garden beet, *B. vulgaris* L. var. spinach, *Spinacia oleracea;* and cells of *Chlorella pyrenoidosa* Chick.

The barley roots were grown in Hoagland's nutrient solution, cut when 20 to 30 mm in length, blotted dry, and treated with gaseous NH_3. Reproducible amounts of gaseous NH_3 were evolved from NH_4OH placed in the stopcock well of Dixon-Keilin flasks at 25° C. The molarity was calculated on the basis of one mole of gas occupying 22.414 liters under standard conditions. In other experiments leaves and root tissues and Chlorella were suspended in buffer and treated with solutions of ammonia. The undissociated ammonia concentration was calculated from the total concentrations of ammonium salt and the pH using a pKb for NH_4OH of 4.74.

Mitochondria were prepared from garden beet roots according to the method outlined by Honda et al (4) and modified slightly. All work was performed at 0 to 3° C with the equipment and solutions precooled at least 12 hours. Plant tissue was cut into approximately two centimeter cubes with a stainless steel knife. The homogenizing medium was a 1.0 M sucrose, with 0.2 M tris (hydroxymethyl) amino methane (tris), and 5×10^{-3} M ethylene diamine tetraacetic acid (EDTA) in redistilled water and adjusted to pH 8 with HCl. One hundred grams of the plant tissue with 100 ml of homogenizing medium were placed in a 1-liter Waring blender. The total blending time was 16 seconds in 4-second intervals 1 minute apart. The debris was removed with the aid of a Pexton laboratory press and the suspension centrifuged at 13,500 to 14,000 × G for 30 minutes to sedimate the mitochondria. The pellet was resuspended in 70 ml of a 0.6 M sucrose, EDTA, tris solution, and again centrifuged at 13,500 to 14,000 × G for 30 minutes. This washed mitochondria pellet was suspended at the rate of 4 ml of 0.6 M sucrose, EDTA, tris per 100 g fresh weight. It was necessary to force the final preparation through a glass wool pad in order to obtain a homogenous suspension. Aliquots of this preparation were taken for nitrogen determinations and subsequently analyzed by the micro-Kjeldahl method.

In mitochondrial studies involving substrate respiration the flask contained the following mixture: 2.0 ml of mitochondrial suspension, equivalent to approximately one milligram of N suspended in 0.6 M sucrose with 25 ppm terramycin according to Freebairn, et al (2), $MgSO_4$ and $MnSO_4$, 5 μmoles; adenosine diphosphate (ADP), 3 μmoles; diphosphopyridine nucleotide (DPN), 3 μmoles; substrate, 45 μmoles; and ammonium salts to provide an undissociated ammonia concentration of 4×10^{-3} M, (12 μmoles) or as indicated. Sufficient 0.2 M tris buffer was added to make a total volume of 3 ml. Results are reported in μl O_2 per hour per flask.

A Beckman Model DU spectrophotometer with a spectral energy recording attachment was used to follow the changes in absorbance of reduced diphos-

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[2] Aided by a grant from the American Cancer Society.

[3] Some of the data reported here are taken from the Ph.D. thesis of H. M. Vines, presented to the Graduate Division of the University of California at Los Angeles, August 1959.

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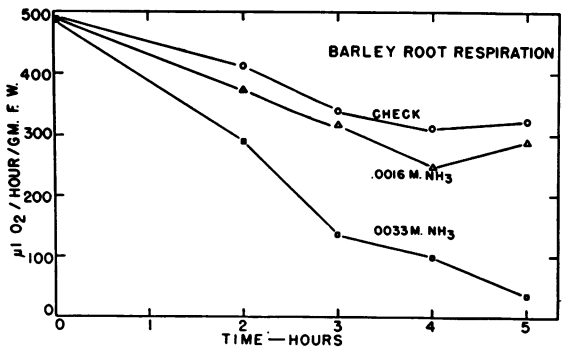
phopyridine nucleotide (DPNH) at 340 mμ (1) as it was oxidized and DPN as it was being reduced by beet root mitochondria. The conditions of these spectrophotometric observations are stated under the corresponding figures.

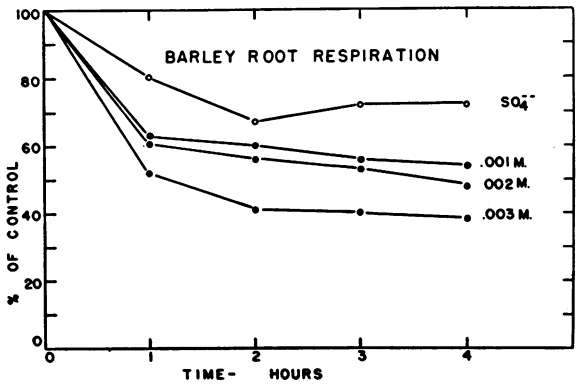
Results

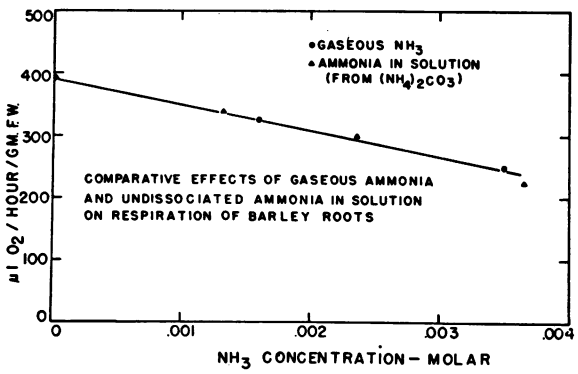
Ammonia Effects on Respiration of Excised Roots and Tissue Slices: A: *Inhibition of endogenous respiration.* Treatment of excised barley roots (blotted dry) with 1.6×10^{-3} M gaseous NH_3 in the flask atmosphere reduced their respiration 23 % and 3.3×10^{-3} M NH_3 caused a 78 % inhibition of respiration after 4 hours (fig 1). Although an effect of NH_3 on respiration of barley roots could be demonstrated in this way, some variability was encountered and the pH of the liquid film in contact with the roots could not be determined. In order to avoid this, solutions of ammonium salts were used. Concentrations of total ammonium salts equal to the gaseous ammonia did not give equivalent respiratory inhibition. Since the amount of inhibition due to ammonium salts was greater at higher pH, it appeared that the undissociated ammonia molecule might be primarily the form which penetrated the cells.

The respiration of excised barley roots treated with $(NH_4)_2SO_4$ solutions containing 1×10^{-3} to 3×10^{-3} M undissociated ammonia in 0.01 M phosphate buffer at pH 7 is shown in figure 2. These concentrations reduced the respiration rate 46 to 62 % within 4 hours after treatment. Although these solutions varied with respect to undissociated ammonia concentrations, they all contained the same concentration of the sulfate ion (0.28 M). The upper line of figure 2 shows that this concentration of sulfate alone inhibited the respiration of barley roots 28 % in 4 hours.

The effect of various other anions and cations on the respiration rate of barley roots at pH 8 was investigated. It was found that a greater inhibition was obtained when ammonia was supplied as the sulfate or chloride salt than when the anion was $CO_3^=$. Other monovalent cations such as K and Na had a







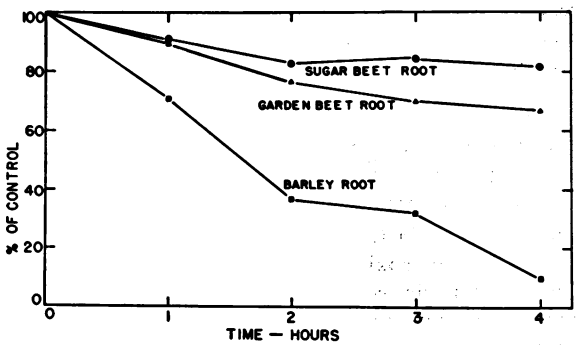


Fig. 1 (*upper left*). Effects of gaseous ammonia on the respiration rate of excised barley roots: △ = 1.6×10^{-3} M NH_3 evolved from 0.1 ml of 0.1 M NH_4OH; □ = 3.3×10^{-3} M evolved from 0.1 ml of 0.2 M NH_4OH; ○ = check.

Fig. 2 (*upper right*). Effect of the sulfate anion and undissociated ammonia on the respiration of excised barley roots. The control roots were in phosphate buffer at pH 7 and $SO_4^=$ was constant in all treatments at 0.28 M.

Fig. 3 (*lower left*). Comparison of the effects of gaseous ammonia and undissociated ammonia in solution on the respiration of excised barley roots at the end of a 2 hour exposure period. Calculation of undissociated ammonia was on the basis of final pH.

Fig. 4 (*lower right*). Effect of 4×10^{-3} M undissociated ammonia in solution at pH 7 on the respiration rate of disks cut from sugar beet roots and garden beet roots and excised barley roots.

slight depressing effect on barley root respiration for periods up to 2 hours, but after 4 hours the rates in the presence of these ions exceeded that of the controls, $NaHCO_3$ resulting in a 22% increase, for example.

Since it seemed possible that the undissociated ammonia molecule rather than the ion was effective in the inhibition of respiration, a direct comparison of the effects of undissociated ammonia in solution with gaseous NH_3 at similar concentrations on the respiration of barley roots was made. For this purpose the excised roots were treated either in the dry condition with an atmosphere containing NH_3 or suspended in buffer with ammonia supplied by $(NH_4)_2CO_3$ as described above. In figure 3 the inhibition of respiration by concentrations of these two forms of ammonia ranging from 1×10^{-3} M to 3×10^{-3} M after 2 hours exposure is shown. The undissociated ammonia in solution was calculated from the final pH at the end of the experiment. The maximum change in pH of the buffer due to a concentration of 0.7 M $(NH_4)_2CO_3$ was a rise from pH 8.0 to 8.26 at the end of 4 hours. These data show a close correlation between the inhibitory effect of undissociated ammonia in solution and NH_3 gas, lending support to the idea that both have a common form which is responsible for the inhibition.

The effect of ammonia upon respiration of garden beet and sugar beet root disks was similar to that on barley roots, although 4×10^{-3} M undissociated ammonia was somewhat less effective in inhibiting the respiration of the beet roots as is shown in figure 4.

B: *Inhibition of exogenous respiration.* The effect of ammonia on oxidation of glycolytic and Kreb's cycle intermediates was studied by infiltrating glucose, citrate, pyruvate, α-ketoglutarate or malate into root tissues. The infiltrated tissues, suspended in 3 ml of 0.2 M tris buffer at pH 8 were treated with ammonia by tipping in an appropriate concentration of $(NH_4)_2CO_3$ 20 minutes after closing the flasks. Figure 5 shows the increase in respiration of barley roots infiltrated with glucose, malate, and pyruvate, and the respiratory inhibition resulting from treatment of these roots with 3×10^{-3} M undissociated ammonia. In this experiment the initial control rate was 460 μl O_2/hr/g F.W. and at the end of 4 hours it was 310 μl O_2/hr/g F.W. In this and other experiments using $(NH_4)_2CO_3$, the first hourly readings for the ammonia treated flasks are omitted as it was found that approximately 30 minutes were required for manometric equilibration of the gases evolved when the ammonium carbonate solution was tipped into buffer containing no plant tissue.

It may be seen in figure 5 that ammonia has not only eliminated any exogenous respiration due to the added substrates, but has also inhibited endogenous respiration to about the same extent as was found in figure 2. Similar results were obtained with citrate and α-ketoglutarate.

In this experiment and in others in which substrates were infiltrated into beet root disks, beet leaf disks, and spinach leaf disks a respiratory response was not always obtained from the substrate, e.g., pyruvate in figure 5. In no case where a substrate gave a response was there any indication that it was being oxidized by a reaction located after a hypothetical metabolic block due to ammonia. To clarify this question and permit respiratory responses from other substrates, mitochondrial preparations were used in additional experiments described later.

CHANGES IN PERMEABILITY DUE TO AMMONIA: The inhibitory effect of ammonia on the respiration of excised roots or tissue slices could be due not only to some general or specific block in metabolism, but also might come about through an effect of ammonia on the integrity of the cell membrane, resulting in a partial disorganization of the cell reflected in a decreased oxygen uptake.

A: *Loss of anthocyanin from beet root disks.* The possibility of an effect on permeability was evaluated by measuring the loss of anthocyanin from garden beet root disks to the bathing medium under the influence of ammonia. Table I shows the change in absorbance of the bathing medium when 20 disks 9 mm in diameter and 1 mm thick were incubated in 10 ml of 0.2 M tris buffer at pH 8 for 2 hours in the

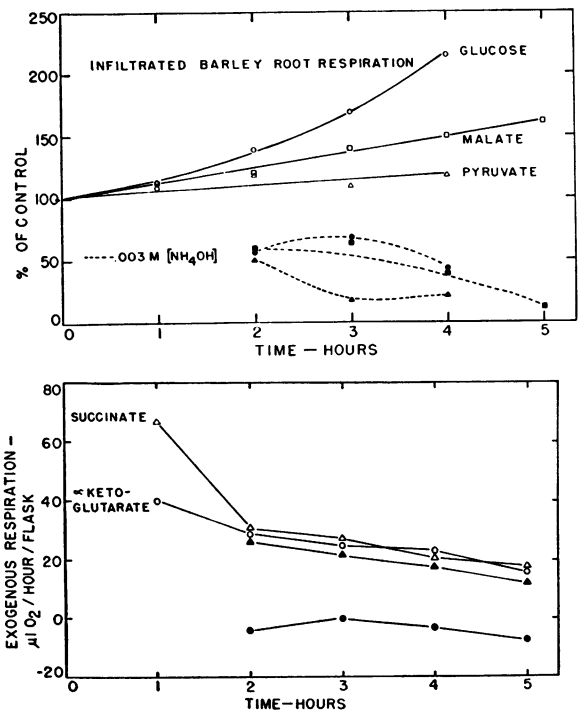


FIG. 5 (*upper*). Exogenous respiration rate of excised barley roots infiltrated with selected substrates at pH 8. Solid symbols indicate companion treatments treated with 3×10^{-3} M undissociated ammonia. Control was infiltrated with tris buffer.

FIG. 6 (*lower*). Rate of oxidation of succinate and α-ketoglutarate in garden beet root mitochondria (open symbols) and the effect of 4×10^{-3} M undissociated ammonia (solid symbols).

TABLE 1

LOSS OF ANTHOCYANIN FROM RED BEET ROOT DISKS TO BATHING MEDIUM FOLLOWING TREATMENT WITH AMMONIA IN SOLUTION

UNDISSOCIATED AMMONIA	ABSORBANCE 410 mμ
Buffer	0.69
0.002 M	0.76
0.004 M	0.77
0.006 M	0.80
0.008 M	0.95
Boil	>2.00

presence of 0.002 M, 0.004 M, 0.006 M, and 0.008 M undissociated ammonia. One set of disks was boiled for 3 minutes to completely disrupt the cell membrane and provide a measure of the maximum possible loss.

It may be seen from this table that 4×10^{-3} M ammonia has increased the loss of anthocyanin from the beet disks by only 6 % of the possible maximum indicated by the boiled tissues, while the same concentration of ammonia caused more than 30 % inhibition of oxygen uptake (fig 2). Twice this concentration of ammonia has brought the loss of anthocyanin up to 20 % of the possible maximum, but damage to cell membranes seems inadequate to explain the observed effects on respiration.

B: *Loss of S^{35} labeled material from Chlorella.* In another attempt to evaluate the effects of ammonia on permeability, Chlorella cells were labeled with S^{35} by being incubated for 4 hours in nutrient solution containing 0.28 M $S^{35}O_4^{=}$. They were washed twice by centrifugation to remove external isotope and incubated for 2 hours with various concentrations of ammonium salt in 0.2 M tris buffer at pH 8. The amount of S^{35} in both the cells and the bathing medium was determined. 4×10^{-3} M ammonia has resulted in even less loss of S^{35} labeled material from the cells than was found in buffer alone (table II).

AMMONIA EFFECTS ON OXIDATIVE ACTIVITY OF MITOCHONDRIA: In order to minimize the problems caused by the presence of a permeability barrier at the cell membrane and to facilitate study of ammonia effects on the oxidation of individual substrates, treatments of garden beet mitochondria were undertaken.

TABLE II

LOSS OF $S^{35}O_4^{=}$ TO BATHING MEDIUM FROM CHLORELLA PYRENOIDOSA INCUBATED FOR 2 HOURS WITH ISOTOPE THEN TREATED FOR 2 HOURS WITH AMMONIA IN SOLUTION

UNDISSOCIATED AMMONIA	% S^{35} EXTRACTION
Buffer	14.7
0.002 M	5.7
0.004 M	6.5
0.008 M	17.8
Boil	75.2

The effect of external pH on the response of mitochondria to ammonia was similar to that observed with intact cells, and indicated that the molecular form of ammonia was also the effective inhibiting agent here.

A comparison of carbonate, sulfate, and phosphate salts of ammonia on the oxidative activity of beet mitochondria showed that equal concentrations of $(NH_4)_2SO_4$ were more toxic than either NH_4CO_3 or $(NH_4)_2HPO_4$ and the carbonate salt was used in mitochondrial studies.

Figure 5 summarizes the effect of 3×10^{-3} M undissociated ammonia on the oxidation of glucose, pyruvate, citrate, and malate by beet root mitochondria where the exogenous respiration of these compounds is entirely eliminated by ammonia within 2 hours.

Similar data for the respiration of α-ketoglutarate and succinate by garden beet root mitochondria are given in figure 6 which shows that although α-ketoglutarate oxidation is completely suppressed by 4×10^{-3} M undissociated ammonia, this same concentration has relatively little effect on succinate oxidation for periods up to 5 hours.

This partial resistance of succinate oxidation to ammonia inhibition seemed to point toward a possible association of DPN with ammonia toxicity, since DPN does not serve as a cofactor for succinate oxidation (5). A study of the effect of ammonia on the oxidation and reduction of DPN by beet homogenates showed that DPNH oxidation was completely inhibited while no effect was found on the reduction step (11). Since the homogenates contain soluble enzymes and cofactors, and high concentrations of ammonia were supplied as a gas, it was desirable to study the reactions under more controlled conditions.

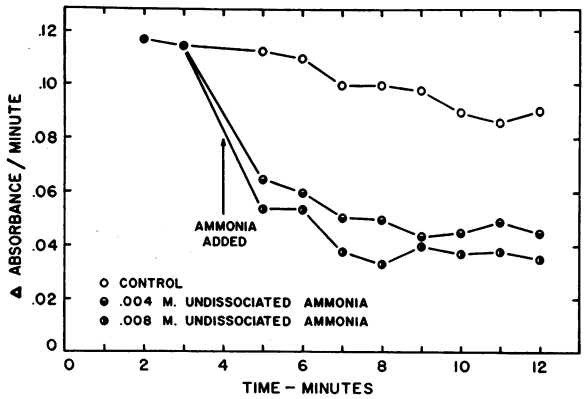
Mitochondria prepared from garden beet roots were used for this purpose. The mitochondria were washed twice in 0.6 M sucrose in 0.2 M tris buffer at pH 8 after being prepared in 1.0 M sucrose with 5×10^{-3} M EDTA and 0.2 M tris buffer at pH 8.

Figure 7 shows the rate of DPNH oxidation by these mitochondria plotted as △ absorbance/minute over a 12-minute period and the effect of 4×10^{-3} M and 8×10^{-3} M undissociated ammonia on this oxidation. The lower concentration caused an inhibition of about 50 % and doubling the concentration increased this inhibition somewhat.

Similar data for the reduction of DPN by mitochondria supplied with citrate and treated with ammonia are given in figure 8. The slight, but significant increase in the rate of DPN reduction caused by ammonia may be related to the inhibition of the reverse reaction, and has not been consistently found in all similar experiments.

DISCUSSION

Because of the alkaline nature of ammonia in solution the pH per se has often been implicated as the basic cause of toxicity to plants resulting from ammonia applications. However, the results of this study indicate that the toxicity results from reactions



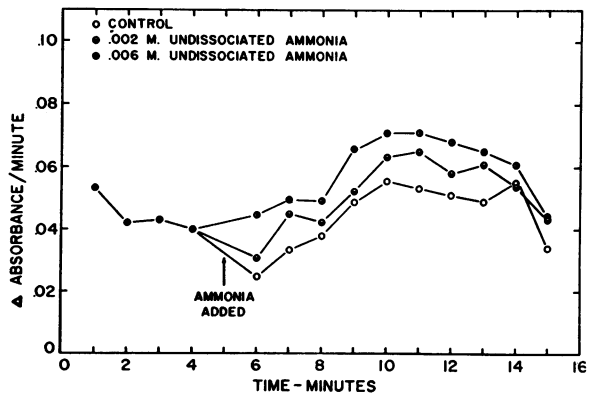


FIG. 7 (*upper*). Effect of ammonia on the rate of oxidation of DPNH in mitochondria plotted as change in absorbance per minute at 340 mμ at pH 8. Each cuvette contained cytochrome c, 0.225 mg; DPNH, 3 μmoles; mitochondria 0.5 ml; appropriate ammonia or buffer and sufficient 0.2 M tris at pH 8 to make a total volume of 3 ml.

FIG. 8 (*lower*). Effect of ammonia on the rate of reduction of DPN in mitochondria plotted as change in absorbance per minute at 340 mμ at pH 8. Each curvette contained DPN, 3 μmoles; citrate, 30 μmoles, appropriate ammonia, or buffer and sufficient 0.2 M tris at pH 8 to make 3 ml.

of ammonia inside the cells and that the primary effect of a high pH in increasing damage to plants from ammonia applied to the soil is probably to cause an increase in the amount of ammonia entering the cell.

The form of ammonia which is an effective inhibitor of respiration in excised plant tissues and mitochondria appears to be the undissociated molecule rather than the ammonium ion. An equivalent concentration of ammonia gas and undissociated ammonia in solution produced the same inhibition of respiration in excised barley roots. With other leaf and root tissues an increased respiratory inhibition was found when the pH of a buffer containing a constant concentration of ammonium salt was raised. If the same concentration of undissociated ammonia is maintained by reducing the pH and increasing the concentration of ammonium salt, the inhibitory effect remains at the same level in either tissue preparations or mitochondria. Whether this relation arises through failure of the ions to penetrate the permeability barriers of cells or particles or whether the molecule itself is active at the site of inhibition cannot be determined from the data available.

The possibility that ammonia was producing inhibitory effects on tissue respiration through a nonspecific effect on permeability of the cell membranes and thus resulting in a loss of organization of the protoplasm, was largely eliminated by demonstration of the effect of ammonia on the permeability of cells was much less than its effect on respiration. However, the possibility of some more specific effect of ammonia on cell permeability remains, and the effect on permeability of mitochondria has not been investigated.

The evidence presented on the inhibition of the respiration of intact cells by ammonia indicates that ammonia blocks metabolism but there is no suggestion as to the location of the block. There is some indication that a fraction of endogenous respiration proceeds by a pathway which is resistant to ammonia inhibition, particularly in beet root disks and spinach leaf disks. The exogenous respiration of glucose, citrate, and malate by barley roots and other plant tissues was inhibited by ammonia in the same way as the endogenous respiration.

These substrates as well as pyruvate, α-ketoglutarate, and succinate were found to give respiratory responses with mitochondrial preparations, but oxidation of all these substrates except succinate was inhibited by ammonia.

Succinate oxidation is known to proceed without the utilization of DPN, although the remainder of the electron transport system is common to succinate and other Kreb's cycle intermediates (1). This fact, coupled with the resistance of succinate oxidation to ammonia treatment indicated that ammonia toxicity might be associated with reduction or oxidation of DPN. Inhibition of DPNH oxidation by ammonia in homogenates has been reported by Wedding et al (11).

The oxidation of DPNH by mitochondria from beet roots was found to be inhibited by either gaseous NH_3 or ammonia supplied in solution via ammonium salts. The reduction of DPN shows either no response to ammonia, or a slight stimulation which may be due to simultaneous inhibition of the reverse reaction.

Work recently reported by Krogmann et al (6) using spinach chloroplast preparations indicated that ammonia is an effective uncoupler of photosynthetic phosphorylation. This may represent a similar interference of ammonia with electron transport in the photosynthetic reaction.

It is suggested that at least part of the toxicity of ammonia is due to its ability to specifically inhibit the oxidation of DPNH and thus block the transport of electrons from oxidized substrates to oxygen.

Summary

The mechanism of ammonia toxicity to plants was investigated. Oxygen uptake in a Warburg respirometer was used as an indication of the effect of gaseous ammonia and ammonium salts on respiration of excised barley roots, garden beet root disks, as well as leaf disks of spinach and sugar beets, and garden beet root mitochondria.

Gaseous NH_3 and undissociated ammonia in equal concentrations were found to inhibit respiration to the same degree in the tissue studied. The pH level was important in ammonia toxicity to the extent that it controlled the undissociated ammonia concentration. Therefore the undissociated ammonia probably is the effective form of ammonia causing inhibition of respiration in plants.

Added substrates were ineffective in overcoming the respiratory inhibition of infiltrated intact tissue or mitochondrial preparations. Substrates studied include glucose, pyruvate, citrate, alpha-ketoglutarate, succinate, and malate. Of these only succinate appeared resistant to ammonia.

The partial resistance of succinate oxidation to ammonia treatment indicated a possible association with the electron transport system since succinate does not require diphosphopyridine nucleotide (DPN) as a cofactor. Studies with DPN reduction and oxidation in a recording spectrophotometer at 340 mμ showed that the oxidation of reduced diphosphopyridine nucleotide (DPNH) was inhibited by ammonia treatment. The companion reaction, DPN reduction, was speeded up following ammonia treatment, probably due to the competition of the two simultaneous reactions.

It is suggested that the site of ammonia toxicity to plants is located in the electron transport system, specifically on the DPNH→DPN reaction.

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